Chapter

Solid-State Anaerobic Microbial Ensilage Pretreatment

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Abstract

Pretreatment technology has become the main bottleneck restricting the development of biogas. This chapter provides an overview of recent studies on solid-state microbial ensilage pretreatment for the production of biogas with wastes. The goal of microbial ensilage pretreatment is to maximize the production of lactic acid, thereby reducing the pH value and establishing an environment that is not suitable for the growth of harmful organisms. The use of various additives, especially lactic acid bacteria, is the main factor to ensure the success of anaerobic pretreatment. Sensory evaluation is carried out by observing the smell, structure, and color of silage to judge the quality of silage. The pH values, ammonia nitrogen, and organic acids (lactic-, acetic-, propionic-, and butyric acid) are used as reference values to determine the fermentation quality of silage. An overall comparison of the effectiveness of microbial ensilage with aerobic microbial pretreatment for biogas production is also discussed. Finally, the research on solid-state anaerobic microbial silage pretreatment in biogas conversion is summarized. The combined anaerobic digestion method with different pretreated materials will be the future development direction due to its advantages.

Keywords: biogas, biomass, ensiling, pretreatment, wet storage

1. Introduction

At present, a large number of organic wastes (such as straw, animal manure, excess sludge, and other wastes) are produced in industry, agriculture, and aquaculture every year. How to deal with organic wastes sustainably has become a global challenge. Anaerobic digestion (AD) technology for the stable utilization of organic wastes (mineralizing volatile solids and reducing pathogens) has been applied all over the world. AD is a biological process in which organic matter is decomposed by an assortment of microbes under oxygen-free conditions and produces biogas (about 50–60% CH_4 and 25–30% CO_2) [1]. Physical approaches, mainly compression and liquefaction, have been commercially applied to upgrade biogas to bio-compressed natural gas (CNG) and liquefied biogas (LBG) [2]. Meanwhile, the nutrient-rich biogas slurry is commonly used as an organic fertilizer [3]. At present, AD with various wastes to produce biogas is the main method to solve the problems of energy shortage and environmental pollution [4]. During the development of AD engineering, the difficulty faced is how to improve the conversion efficiency of waste. Various wastes have unstable AD and low conversion efficiency due to structural composition and nutritional imbalance. The agricultural waste is closely bound together by the covalent bonds among its main components cellulose, hemicellulose, and lignin, which greatly hinders the degradation of carbohydrates [5]. Especially for corn stover, it found that the index changed after the straw was dried, which was very unfavorable for biogas fermentation and other bioenergy conversion methods. For example, the lignin content is almost doubled, which seriously hinders the degradation of cellulose and hemicellulose in the process of biochemical transformation. AD will lead to problems such as slow start of fermentation, long fermentation time, and low gas production rate. Therefore, pretreatment becomes a necessary step for AD of lignocellulose. In the field of biomass transformation, the digestibility of cellulose is affected by many factors, such as hemicellulose content, lignin characteristics (content and distribution), matrix-specific surface area and porosity, cellulose crystallinity, and cell wall thickness [6]. The purpose of pretreatment is to remove or destroy the complex structure between cellulose-hemicellulose-lignin, improve the effective contact between cellulase and biomass, and then increase the rate of enzymatic hydrolysis [7, 8]. So far, scientific researchers in various countries have developed many promising pretreatment technologies, but most of them are chemical methods that require the addition of chemical reagents, such as acid, alkali, ammonia, organic solvents, or ionic liquids, and require certain high-temperature conditions and corresponding special reaction equipment [9–12]. The effect of different pretreatment methods is different, resulting in a great difference in the final gas production situation.

Biological pretreatment is to destroy the cell wall structure of biomass by the metabolic activity of microorganisms, which has the characteristics of mild use conditions, low cost, and has great potential in the field of biomass pretreatment [13]. Compared with chemical methods, biological methods do not need to consume a large amount of energy and recover chemical reagents, nor produce harmful inhibitors in the reaction system [14]. In parallel, microbes have evolved mechanisms, including cellulose-degrading enzymes, to degrade plant cell walls to access the plants' nutritious sugars. Fungi (aerobic pretreatment) use degradative enzymes called cellulases, whereas in bacteria (anaerobic pretreatment), multiple enzymes self-assemble into a complex called the cellulosome [15]. Both biological pretreatment methods have their advantages and disadvantages. Therefore, the main purpose of the chapter was to provide an overview of recent studies on solid-state microbial pretreatment for the production of biogas with wastes, focusing on the steps involved in the anaerobic ensilage formation with microbial ensilage pretreatment, additives, and quality evaluation of the silage.

2. Aerobic microbial pretreatment

From both economic and environmental perspectives, fungal pretreatment with lignin-degrading microorganisms, preliminary white-rot fungi, has received renewed interest as an alternative to thermal/chemical pretreatment for biogas production [16]. The degradation of lignin by white-rot fungus is a process of biological oxidation. Under suitable conditions, the mycelium of white-rot fungus first secretes super fiber oxidase to dissolve the wax on the plant surface, and then the mycelium enters the plant interior and secretes the enzyme system to degrade lignin to complete the degradation of lignin. The results show that white-rot fungus can not only degrade lignin but also protect cellulose from damage, so as to improve the quality of lignocellulose and make it easier to be degraded by anaerobic bacteria. Ghosh and Bhattacharyya used *Phanerochaete chrysosporium* and *Polyporus ostreiformis* to treat rice straw, by which biogas and methane production was increased by about 34.73 and 46.19% in treated straw, respectively [17]. Taniguchi et al.

compared the gas production effect of four kinds of white-rot fungi (*Trametes versicolor*, *Phanerochaete chrysosporiu*, *Ceriporiopsis subvermispora*, and *Pleurotus ostreatus*) on the pretreatment of rice straw. It was found that *P. ostreatus* selectively degraded the lignin fraction of rice straw rather than the holocellulose component [18]. Zhong et al. analyzed the pretreatment effect of *Pleurotus florida* on anaerobic fermentation of corn straw to produce biogas. AD experiments showed that the biogas productivity was increased by all the pretreatments and that the biogas production after NaOH pretreatment would be 20.07 and 16.58% higher than the raw corn straw and biologically pretreated ones, respectively [19]. Mackulak et al. used *Auricularia auricula-judae* to pretreat sweet chestnut leaves and hay at 37°C for 4–5 weeks, which had a 15% increase in biogas production compared with the untreated samples [20].

The advantages of this technology over thermochemical pretreatments include simple techniques, low energy requirements, no or reduced output of waste streams, reduced downstream processing costs, and no or reduced inhibitors to biogas fermentation. Despite the advantages, substantial holocellulose loss and long pretreatment time are the major issues associated with fungal pretreatment. The growth cycle of microorganism is long, such as white-rot fungus, which usually takes 7–15 days generally. White-rot fungi are sensitive to temperature, which grow faster at 26–32°C, and their growth will be inhibited if the temperature is too high or too low. Meanwhile, compared with chemical and physical methods, the biodegradation efficiency of lignocellulose by biological method is not high, while the pH value and material composition will also affect the growth of microorganisms. For example, white-rot fungus grows better under the condition of partial acid, and its growth and enzyme activity will be hindered with the increase in pH value. In addition, there are a few kinds of microorganisms that could degrade lignin, while the low enzyme activity is also an important factor limiting its application.

3. Anaerobic microbial pretreatment

Microbial ensilage under anaerobic conditions can be a good way to avoid the above problems of aerobic pretreatment, which become a reliable method for long-term storage of lignocellulosic biomass (LCB) [21]. Its basic principle is to use the anaerobic fermentation of lactic acid bacteria (LAB) in closed conditions to convert the soluble carbohydrates to organic acids, which inhibit the growth of detrimental microorganisms by a strong drop in pH to values between 3 and 4 [22]. After a year of microbial ensilage, the dry matter (DM) loss of raw materials is as low as 1–5%, while the digestibility is higher than that of dry storage [23], mainly because the degradation of non-structural carbohydrates reduces the biological resistance of LCB [24]. Microbial ensilage can solve the problems of straw collection, preservation, and pretreatment in large scale. Compared with the chemical methods, the technology does not need to consume a lot of energy and recover chemical reagents, nor produce harmful inhibitors in the reaction system. To date, the silage remains one of the main methods for the efficient utilization of LCB.

3.1 The steps involved in the anaerobic ensilage formation

High-quality silage begins with harvesting at an appropriate stage of maturity to maximize the nutrient production. Next, the following management methods are essential for the successful fermentation and preservation of product (**Figure 1**).

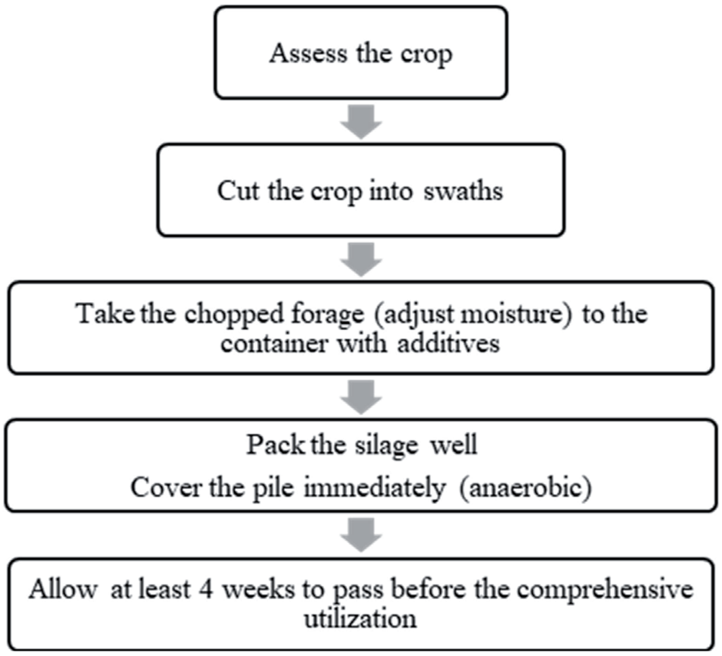

Figure 1.
The pictorial presentation of the steps involved in the ensilage formation.

Figure 2.
Large-scale storage in movable microbial ensilage membranes.

The goal of good fermentation is to maximize the production of lactic acid, thereby reducing the pH value and establishing an environment that is not suitable for the growth of harmful organisms. Good silage is made with less exposure to the air, allowing the *Lactobacillus* to start fermenting quickly. For example, modern biogas engineering adopts the following pretreatment devices to complete the anaerobic fermentation process (**Figure 2**).

According to the main biochemical and microbiological transformations occurring during the process, ensiling can be divided into three phases as follows.

1. Aerobic fermentation process

Anaerobic microbial pretreatment is a straw pretreatment technology utilizing microbial fermentation under anaerobic conditions. However, in the process of straw closure, there is more or less oxygen in the straw raw materials, which makes aerobic microorganisms grow and propagate in the first few days of fermentation. Through the activity of these aerobic microbes, a small amount of sugar and oxygen in the straw can be converted into carbon dioxide and water.

Finally, the oxygen becomes less and less until the oxygen content drops to zero. Aerobic microorganisms do not survive.

2. Enzymatic hydrolysis process

Due to the activity of microorganisms, various enzymes are produced, which destroy the structure of cellulose, hemicellulose, and lignin in the straw and make them degrade step by step to form various sugars. The enzymatic hydrolysis process of straw is relatively slow. With the increase of microbial reproduction and the improvement of microbial activity, the straw is gradually enzymatically hydrolyzed into sugars. Hemicellulose is most easily degraded throughout the enzymatic hydrolysis proofreading, while a larger amount of xylose, arabinose, glucose, mannose, and galactose is formed. When these sugars reach a certain concentration, microorganisms can use these sugars as substrates for acid production and fermentation.

3. Acidogenic fermentation process

Microorganisms utilize sugars in the straw as substrates and convert them into organic acids. After aerobic fermentation of the straw, oxygen is exhausted and aerobic microorganisms cannot survive. Under anaerobic conditions, anaerobic microorganisms cannot completely convert sugar substrates into water and carbon dioxide, but can only be decomposed into various organic acids, including hexanoic acid, propionic acid, lactic acid, and butyric acid. These organic acids are ionized in the straw to form a large amount of hydrogen ions, which acidify the straw and decrease the pH value. When the pH value drops to 4.5–5.0, acidity inhibits the activities of various microorganisms, thus slowing down the microbial activities and forming a good straw silage.

3.2 Additives

In addition to a small amount of LCB in raw materials, there are also a large number of spoilage bacteria, yeasts, and other flora that are not conducive to silage fermentation. Most of these floras are aerobic bacteria, while lactobacilli belong to anaerobic bacteria. In the early stage of fermentation, the LCB begin to propagate only after the oxygen in silage is consumed by these aerobic mold spoilage bacteria to form an anaerobic environment. In this process, silage will cause fever and serious occurrence of earthy or mildew taste, which are all adverse factors from silage. Artificial addition of additives can shorten the process, which makes LCB become the dominant bacteria group of silage and improve the overall quality of silage. At present, there are more than 200 silage additives used all over the world, which can be classified into four categories as a whole, namely, bacterial inoculants, enzyme, non-protein nitrogen, and preservatives.

3.2.1 Bacterial inoculants

LAB plays a major role in the fermentation process of silage, whose main strains include *Lactococcus* (*Streptococcus faecalis*, *Staphylococcus lactis*, *Lactobacillus lactis football*, etc.) and *Lactobacillus* (*Lactobacillus plantarum*, *Lactobacillus brucei*, *Lactobacillus casei*, *Lactobacillus brevis*, etc.). Inoculated LAB in silage can be divided into two types: homozygous and heterotypic fermentation. Homotypic fermentation mainly includes *Lactobacillus plantarum* and *Pediococcus lactis*, while heterotypic fermentation is mainly *Lactobacillus brucei* at present. The advantages of homozygous fermentation are that one glucose is converted into two lactic acids, which have strong acid-producing ability, fast pH value reduction, and less DM loss during fermentation. However, its disadvantage is that it produces a large amount of lactic acid, resulting in poor aerobic stability after cellar opening. After exposure to air, the silage pH value increases rapidly and the silage temperature increases accordingly. Heterotypic fermented convert 1 mol of glucose into 1 mol of lactic acid and 1 mol of acetic acid. Because the acidity of acetic acid is less than that of lactic acid, the speed of reducing pH value in fermentation is slower than that of the same type, and the loss during fermentation is higher than that of the same type of LCB. However, due to the production of a large amount of acetic acid, the aerobic stability after cellar opening is very good, up to 100 hours.

Lactobacillus inoculum, or LAB starter, is widely recognized and commercialized, mainly because it can reduce the loss of DM in the early stage of fermentation. However, during silage opening, this microbial inoculum failed in preventing thermal deterioration of silage and did not improve the aerobic stability of silage. What bother producers is certain yeasts that have a strong utilization of lactic acid, which are insensitive to high concentrations of lactic acid, are highly tolerant to low pH environments, and cannot inhibit its activity in acidic environments. They grow well in the acidic environment of silage. When the silage is opened, they are twice as active after exposure to air as in the sealed environment of silage. During the period of aerobic exposure, these yeasts can utilize lactic acid as an energy substance. The aerobic activity of yeasts will lead to the increase of silage pH due to the utilization and consumption of lactic acid, which weakens the acidic environment. In such an environment, other aerobic bacteria and molds will also activate, while the degree of silage corruption will deepen. Ultimately, the activity of aerobic microorganisms can not only lead to the loss of sugar, starch, and protein but also produce a lot of heat because of the large number of microbial reproduction and activity.

Nowadays, widely used and recognized inoculants contain mixed microbial inoculants of both homotypic LAB (to reduce pH) and heterotypic *Lactobacillus brucelli* (to inhibit yeasts), which can ensure both successful fermentation during anaerobic fermentation of silage and reduction of fever, deterioration, and spoilage during open pit.

3.2.2 Enzyme

Additional enzymes mainly refer to a variety of cell wall degrading enzymes, such as cellulase, hemicellulase, amylase, and pectinase. The purpose of adding enzymes is to reduce the fiber content in silage. In addition, more sugars can be degraded by enzymes for LAB fermentation. As enzymes contribute to the degradation of acidic and neutral washing fibers, lactic acid fermentation, dry matter recovery, and storage life are improved. Because of the large demand and high cost of enzyme preparation in the process of adding, it is seldom used in the actual production process.

3.2.3 Antiseptic additives

In order to reduce the pH value quickly, soften the silage, and facilitate the digestion of materials, dilute sulfuric acid or hydrochloric acid can be added to the silage. Preservatives are added to make the silage sink quickly, compact easily, increase the storage capacity, and make the silage crops stop breathing (biological oxidation) quickly, thus improving the success rate. Formic acid and propionic acid are commonly used. Benzoic acid and its sodium salt also have good bacteriostatic effect on molds in silage with dosage not exceeding 0.1%. In the United States and other places, calcium formate plus sodium sulfite was used for silage. You can also choose some other antifungal agents, such as sorbic acid and its potassium salt, as appropriate, but the price is higher. When using mold inhibitors, spray them evenly on the shredded silage raw materials as far as possible, compact them in layers, and seal them in good condition.

3.3 Quality evaluation of the silage

The standardization and development of silage quality grading may help to accurately assess the quality, which is one of the key measures to improve the quality of forage. Generally speaking, quality evaluation of silage includes sensory evaluation and chemical analysis [25]. Sensory evaluation is simple and feasible, but subjective; laboratory evaluation of chemical analysis involves many indicators, which can be quantitatively compared objectivity. According to the color, odor, texture, structure, and other indicators of silage, the quality of silage was evaluated by sensory operation. Chemical analysis evaluation is to quantitatively evaluate the chemical composition of silage through instrumental analysis, while the main indicators are pH value, organic acids (lactic acid, acetic acid, butyric acid, etc.), ammonia nitrogen, total nitrogen, alcohols, and other fermentation parameters.

3.3.1 Sensory evaluation

Using the sensory evaluation method (**Table 1**), we can quickly and intuitively judge whether the silage material is deteriorating.

The author has carried out anaerobic pretreatment on dry corn stover (**Figure 3**). It can be seen that after 28 days of storage, there is no mildew in the samples of dry corn stover silage. The color of the silage material is yellow and has a strong acid flavor, indicating that the process was successful.

3.3.2 Laboratory measurements of the silage

1. **Lactic acid**: the goal of good fermentation is to maximize the production of lactic acid.

   Lactic acid is the strongest fermentation acid in the fermentation process, which has the best effect in reducing pH value. Rapid reduction of pH value helps to reduce protein decomposition, increase the acidolysis of hemicellulose, and reduce other useless microbial activities. The high ratio of lactic acid and lactic acid/acetic acid indicates that good fermentation has taken place.

Sensory evaluation	Quality		
	High	**Medium**	**low**
Odor	Sour smell, aroma, and strong sour taste, like distillers' grains and pickles	Less acidity and fragrant but not strong flavor	Strong butyric acid or pungent odor, mildew odor
Color	Green or yellow-green, similar to the color of the raw material	Yellowish brown or dark green	Brown or black
Texture	Stems and leaves are well preserved, and the hands are loose, soft, and slightly moist	Soft but slightly dry or slightly watery, with separable stems and leaves	Stems and leaves rot, stick together, or are loose, dry, and hard

Table 1.
The sensory evaluation of silage.

Figure 3.
The surface morphology of the starting material and the resulting silage.

2. **Acetic acid**: the formation of acetic acid usually occurs in the first 2 to 3 days of silage.

When the pH value drops below 5.00, the *Lactobacillus* begins to grow and takes over the fermentation process. Therefore, in typical silage, the production of acetic acid is helpful to activate the production of lactic acid. In the process of opening cellar to take silage, the degradation of lactic acid by yeast can also produce acetic acid. The concentration of acetic acid is usually low (<3%). It has some antifungal properties, which help to prolong the retention time of the silage.

3. **Propionic acid**: usually found in only a small proportion with silage.

A high level of propionic acid with silage indicates a large error in some places. It is normal that propionic acid is higher when adding propionic acid additive.

4. **Butyric acid**: butyric acid is produced by *Clostridium perfringens*. If the silage is too wet (DM <30%), the bacteria will multiply.

In wet silage, the acid produced by *Lactobacillus* may not be enough to reduce the pH value to prevent the growth of *Clostridium*. *Clostridium* can also ferment lactic acid to produce butyric acid and decompose amino acid to produce excessive ammonia. Both of these effects lead to an increase in pH and further deterioration of silage.

The results of the author's study are as follows: The pH value (4.22), pretreatment time (4 weeks), and the content of lactic acid in dry corn stover silage (4.32%) should be considered as important indicators of the success of microbial ensiling [26].

4. Bioconversion of silage to biogas

In exploring the effect of pretreatment with wastes, many studies have explored the structure and hydrolysis degree of pretreated materials, which as an important index to evaluate the advantages and disadvantages of pretreatment [27]. Like physical and chemical pretreatment, biological pretreatment can also loosen the structure of lignocellulose (**Figure 4**), but it cannot be ignored that the change of lignocellulose structure is only a part of the indicators to evaluate the advantages and disadvantages. It is also an important part of the transformation ability of substrates and the type of metabolites. Various organic acids, especially lactic acid, produced by microbial metabolism during anaerobic pretreatment are important intermediates in

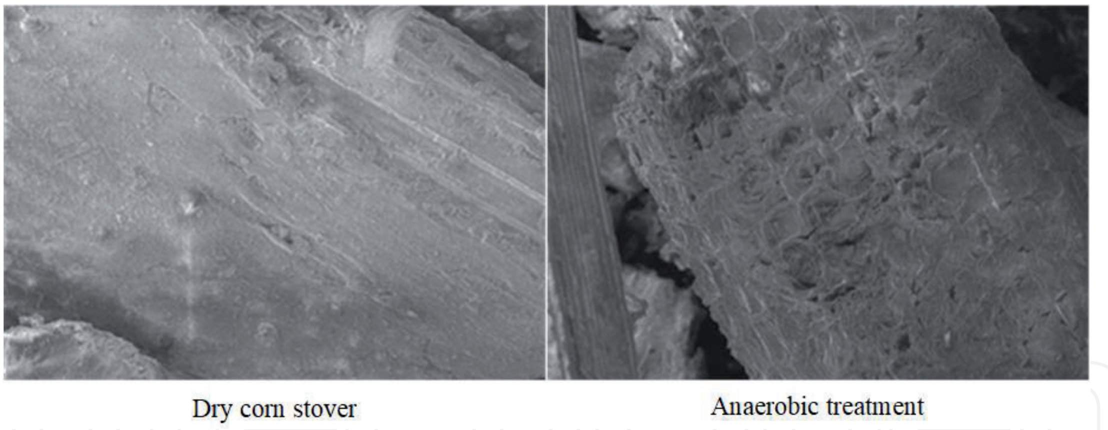

Figure 4.
Scanning electron micrographs of non-pretreated and pretreated dry corn stover.

the AD process [28], which can improve the biogas yield and accelerate the process of biogas fermentation [29]. In addition, the organic acids can effectively neutralize the ammonia and other basic substances accumulated during AD to ensure that the pH value of the reaction system is maintained in a stable range [30]. This indicates that the bio-pretreatment method which can metabolize organic acids can effectively improve the biogas yield. However, not all biological pretreatments can convert LCB into organic acids in straw, or only have low conversion capacity, which may limit the significant improvement of subsequent gas production [31].

In the process of AD, raw materials are not only digestive substrates but also the source of nutrients for the survival of anaerobic microorganisms. The character of raw materials determines the time and the biogas yield of AD [32]. AD is a coordinated and mutually restricted metabolic process among starch, protein, fat, and lignocellulose. The types and quantities of raw material organic matter play a decisive role in the degradation process, raw material utilization efficiency, and biogas yield of AD. For example, AD with single LCB has the problem of imbalance between carbon and nitrogen. The C/N ratio of straw stalk is close to 70:1, whereas the best C/N ratio for AD is 20–30:1 [33]. Due to the low C/N ratio, ammonia nitrogen inhibition occurs in AD with single pig manure, which makes it difficult to form the optimal growth state required by biogas-producing microbiota [34]. Hydrolysis pretreatment is a limiting step, because the structure of the cell wall of the residual sludge can inhibit the hydrolysis of intracellular degradable substances. Gas production from conventional sludge digestion often requires a longer residence time [35].

To solve the above problems, mixed pretreatment with difficult and easy to decompose organic matter is one of the hotspots in the field of AD in recent years. Mixed AD can not only effectively regulate the nutritional balance of single raw materials but also improve the bioconversion rate of materials [36]. The author carried out anaerobic microbial ensilage pretreatment using dry corn stover mixed with pig manure and residual sludge, respectively, and then investigated the biogas yield on this basis. The gas production rate from total solids (TS) of untreated dry straw was only 296 mL/g, and the gas production index has been greatly improved through mixed microbial ensilage. The effect of the dry corn stover and pig manure microbial ensilage pretreatment was the best, whereby the gas production rate of TS reached 599 mL/g, and the average volumetric gas production rate was 0.86 L/(L·d). The AD of corn stover, excess sludge, and pig manure can be used to alleviate the nitrogen limitation when using silage as the main raw material. Broad bioconversion of such raw materials will play a decisive role in solving the problems of burning straw, residual sludge landfill, and non-point source pollution from livestock and poultry manure.

The combined AD of different silage materials has the following points:

1. adjusting the organic nutrients in the fermentation substrate;

2. producing organic acids and intermediate metabolites such as ammonia nitrogen can undergo partial neutralization reaction to maintain the stability of pH value in the process and promote the smooth progress of AD; and

3. increasing the degradation rate of raw materials to shorten the whole fermentation time.

5. Conclusions

Solid-state anaerobic microbial ensilage pretreatment is one of the effective means to improve the biogas yield of raw materials. Other pretreatment methods only change the composition and structure. Biological pretreatment can not only change the composition and structure but also have the ability to ferment and metabolize decomposed substrates into other small molecules, and change the composition and structure. It takes a certain time for fermentation to metabolize small molecule nutrients; so, exploring the optimal pretreatment time can improve the efficiency. Because of the particularity of biological pretreatment, it is also of great significance to explore its impact on the structure, activity, and functional expression of microbial community in the subsequent AD. Understanding the impact of biological pretreatment from multiple angles can better optimize the biological pretreatment conditions, thereby improving the efficiency and enhancing the gas production efficiency for biogas engineering.

The high efficiency of physical pretreatment is accompanied by high energy consumption, and chemical pretreatment is also facing environmental pressure. Biological pretreatment also needs to constantly improve its pretreatment efficiency. Therefore, more and more studies began to focus on mixed pretreatment, that is, to absorb the advantages of different pretreatments to make up for the shortcomings, to achieve a higher hydrolysis rate while reducing energy consumption and reaction time, and to reduce pretreatment costs and environmental hazards. For mixing pretreatment with different materials, screening suitable microbial communities will be a future study direction to ensure a controllable and stable treatment process.

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Conflict of interest

The authors declare no conflict of interest.

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